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Research Paper

The effect of levomepromazine on the healthy and injured developing mouse brain – An *in vitro* and *in vivo* study

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ABSTRACT

Levomepromazine (LMP) is a phenothiazine neuroleptic drug with strong analgesic and sedative properties that is increasingly used off-label in pediatrics and is being discussed as an adjunct therapy in neonatal intensive care. Basic research points towards neuroprotective potential of phenothiazines, but LMP's effect on the developing brain is currently unknown. The aim of the present study was to assess LMP as a pharmacologic strategy in established neonatal *in vitro* and *in vivo* models of the healthy and injured developing mouse brain. *In vitro*, HT-22 cells kept exposure-naïve or injured by glutamate were pre-treated with vehicle or increasing doses of LMP and cell viability was determined. *In vivo*, LMP's effects were first assessed in 5-day-old healthy, uninjured CD-1 mouse pups receiving a single intraperitoneal injection of vehicle or different dosages of LMP. In a second step, mouse pups were subjected to excitotoxic brain injury and subsequently treated with vehicle or LMP. Endpoints included somatometric data as well as histological and immunohistochemical analyses. *In vitro*, cell viability in exposure-naïve cells was significantly reduced by high doses of LMP, but remained unaffected in glutamate-injured cells. *In vivo*, no specific toxic effects of LMP were observed neither in healthy mouse pups nor in experimental animals subjected to excitotoxic injury, but body weight gain was significantly lower following higher-dose LMP treatment. Also, LMP failed to produce a neuroprotective effect in the injured developing brain. Additional studies are required prior to a routine clinical use of LMP in neonatal intensive care units.

Introduction

Worldwide, an estimated 15 million babies are born preterm every year, with rising numbers in many countries. Complications of prematurity cause more than 1 million deaths per annum (Liu et al., 2016), and many survivors face long-term complications and often lifelong disability, especially if brain injury occurs (Luu et al., 2009). The predominant type of brain pathology in preterm infants is a disruption of cerebral white matter in combination with neuronal-axonal damage (Volpe, 2009). The etiopathogenesis of this so-called "encephalopathy of prematurity" is complex (Thornton et al., 2012). Hypoxic/ischemic events in the presence or absence of systemic infection/inflammation (du Plessis and Volpe, 2002) are thought to be main mechanisms setting off the injurious cascade (Volpe, 2001). Excitotoxic cell death, induced by excessive release and impaired re-uptake of glutamate in energy depletion situations, is regarded as a pivotal downstream mechanism (Lau and Tymianski, 2010). In general, perinatal brain injury does not result from a single event, but develops and evolves over time (Thornton et al., 2012). The complex nature of injury calls for treatment strategies that act on multiple levels without interfering with developmental processes.

To date, treatment is confined to supportive care. But especially the sickest and most immature preterm infants are likely to be exposed to multiple stressors during their Neonatal Intensive Care Unit (NICU) stay (e.g. maternal separation, ambient noise, disruption of light-dark cycle, repeated painful procedures) (Stevens et al., 2003), which in turn leads to a "multiple-hit" phenomenon. Particularly pain causes high levels of excitotoxic glutamate and subsequently leads to irreversible neuronal

Abbreviations: bw, body weight; CCK-8, Cell Counting Kit-8; i.c., intracranial; i.p., intraperitoneal; IQR, interquartile range; LMP, levomepromazine; NaCl, sodium chloride; NICU, Neonatal Intensive Care Unit; Px, postnatal day x; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

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injury (Vinall and Grunau, 2014). Pain management in neonates includes different non-pharmacological and pharmacological approaches. Medications administered in NICUs are generally used off-label, with safety data stemming from adult populations or older children (Hall and Shbarou, 2009). Bearing in mind the specific developmental state of preterm infants, caution is warranted: There is reason for concern that drugs currently used for sedation and analgesia in the NICU may have pro-apoptotic effects on the developing brain (Ikonomidou et al., 2001). These effects may be aggravated in case of underlying disease. Alternatives that are safe to use in both healthy preterm infants and those afflicted by brain injury are direly sought for.

Levomepromazine (LMP), also known as methotrimeprazine, is a phenothiazine neuroleptic drug with strong analgesic and sedative effects, which is commonly used in palliative and end-of-life care (Dietz et al., 2013). It is also used off-label as an add-on sedative in Pediatric Intensive Care Units, as it appears to have no major side effects (van der Zwaan et al., 2012; Hohl et al., 2013; Snoek et al., 2014). With regard to brain injury, various phenothiazines proved to be neuroprotective due to their anti-excitotoxic, anti-apoptotic and anti-oxidative properties (Geng et al., 2017; An et al., 2017; Varga et al., 2017; Gonzalez-Munoz et al., 2010). The neuroprotective effect of LMP is a widely under-researched topic, with only two studies showing beneficial effects via inhibition of lipid peroxidase and scavenging reactive oxygen species *in vitro* (Hadjimitova et al., 2002; Jeding et al., 1995). Data on the effects of LMP in the newborn brain are lacking.

The aim of the study was to assess LMP as a pharmacologic strategy in established neonatal *in vitro* and *in vivo* models in a step-wise approach. First, LMP effects in the uninjured brain were evaluated. In a second step, its effects on the injured brain after an excitotoxic insult were investigated.

Experimental procedures

Materials for in vitro studies

DMEM high glucose (4.5 g/L) with glutamine (BE12-604 F), HEPES pH 7.4 (BE17-737E) and Penicillin/Streptomycin (DE17-602E) were purchased from Lonza (Cologne, Germany). Fetal Bovine Serum (FBS HyClone, SH30070.03) was obtained from GE Healthcare Europe (Vienna, Austria), the cell viability assay Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies Japan (CK04, Dojindo EU, Munich, Germany). Cell culture expendables were purchased from Falcon (Corning, Germany). Glutamic acid was purchased from Sigma (G5513, Vienna, Austria), LMP (Neurocil®) was purchased from Bayer Pharma (Leverkusen, Germany).

In vitro experiments

The murine hippocampal neuronal cell line HT-22 was kindly provided by Dr. Jan Lewerenz (Department of Neurology, University of Ulm, Germany). Cells were cultured in DMEM with high glucose supplemented with 10 % heat-inactivated (56 °C, 30 min) fetal bovine serum, 10 mM HEPES, 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified incubator with 5 % CO_2 atmosphere at 37 °C.

For experiments, 5000 HT-22 cells suspended in 100 µL per well were seeded in a 96-well plate and were allowed to settle for 24 h. At a confluency of 50 %–60 %, cells were treated with five different concentrations of LMP (0.05 µM, 0.5 µM, 10 µM, 20 µM and 50 µM) for 30 min at 5 % CO_2/37 °C. Subsequently, they were either returned to standard medium or were exposed to 300 µM of glutamate for 17 h. Untreated, unexposed cells cultivated under standard conditions served as a viability control. Cell viability was assessed using CCK-8 following the manufacturer's protocol. In brief, 10 µL per well of CCK-8 reagent were added and incubated for 2 h at 5 % CO_2/37 °C. Absorbance was measured at 450 nm using a microplate reader (Hidex Sense Microplate Reader, HVD Life Science, Vienna, Austria). Complete medium without cells served as a blank. All samples were analyzed in quintuplicates. Findings were confirmed in four independent subsets of experiments.

Materials for in vivo studies

Ibotenic acid (ibotenate) was purchased from Tocris Bioscience (RD-0285/5, R&D Systems, Germany), isoflurane from Abbvie GmbH (B506100017, Vienna, Austria). LMP (Neurocil®) was obtained from Bayer Pharma (Leverkusen, Germany), cresyl violet acetate from Sigma (C5042, Vienna, Austria). Rabbit polyclonal anti-cleaved caspase-3 antibody was purchased from Cell Signaling Technology (9661, Frankfurt/Main, Germany), biotinylated isolectin B4/G. simplicifolia was obtained from Vector Laboratories (B-1205, Szabo-Scandic, Vienna, Austria), biotinylated goat anti-rabbit IgG from Jackson ImmunoResearch Europe Ltd. (111-065-003 Cambridgeshire, UK), Vectastain Elite ABC Kit from Vector Laboratories (PK-6100 Szabo-Scandic, Vienna, Austria). In situ cell death detection kit, POD (11684817910, TUNEL), recombinant DNase I (04536282001) and proteinase K (03115879001) were obtained from Roche Diagnostics (Mannheim, Germany), diaminobenzidine was purchased from LifeTechnologies (750118, ThermoFisher Scientific, Vienna, Austria), HistoGreen from Linaris (E109, Dossenheim, Germany). Gelatine from cold water fish skin was obtained from Sigma (G7765, Vienna, Austria), biotin-free bovine serum albumin was purchased from Carl Roth (0163.1, Karlsruhe, Germany) and Target Retrieval Solution was from Dako (S1699, Agilent Technologies, Vienna, Austria).

In vivo experimental setup

All animal studies were conducted in compliance with current EU legislation (Directive 2010/63/EU revising Directive 86/609/EEC) and Austrian law. Formal approval to conduct the experiments described has been obtained in advance from the animal subjects review board of our institution (Project number BMWFW-66.011/0158-WF/V/3b/2014). CD-1 mice (Crl:CD1 (ICR), Charles River Laboratories, Sulzfeld, Germany) were bred and kept at the Central Laboratory Animal Facility, Medical University of Innsbruck, Austria, using standard housing conditions with a 12 -h light-dark cycle, at a room temperature of 25 °C and a humidity of 75 %. Animals of both sexes with a body weight $\geq$ 3 g on postnatal day five (P5) were used for the study. All efforts were made to minimize the number of animals used and their suffering.

Somatometry

Body weight (grams) was determined in each animal by means of calibrated medical precision scales from P5 onwards on a daily basis until endpoint determination. Rapid weight loss (>10 % within 24 h) was defined as humane stop criterion. Brain weight (grams) was measured immediately after sacrification on P6 or P10.

Toxicity study in healthy animals

In order to rule out a potential neurotoxic effect of LMP, 5-day-old healthy, uninjured mouse pups received a single intraperitoneal (i.p.) injection of either i) vehicle (sodium chloride, NaCl 0.9 %), or LMP in a dosage of ii) 0.05 µg/g body weight (bw), iii) 0.1 µg/g bw, iv) 0.5 µg/g bw, or v) 1 µg/g bw.

On P6, brains were harvested, formaldehyde-fixed, paraffin-embedded, and sliced serially into 10 µm-thick coronal sections. DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay according to manufacturer's instructions (Griesmaier et al., 2012). In brief, after deparaffinization and rehydration, sections were pretreated with 10 µg/mL proteinase K for 20 min at room temperature for antigen retrieval. In order to quench endogenous peroxidase activity, sections were incubated in 2 % H_2O_2/methanol for 5 min at room temperature. Blocking of

unspecific binding sites was achieved using biotin-free 3% bovine serum albumin in 1x PBS for 30 min at room temperature. Slides were incubated with the TUNEL reaction mixture (enzyme solution and label solution blended 1:10) for 60 min at 37 °C. After several PBS rinses, slides were incubated with a peroxidase-conjugated anti-fluorescein antibody for 30 min at 37 °C. Visualization was performed using HistoGreen as a chromogen. Following various rinses in distilled water, sections were dehydrated, cleared in xylene and cover-slipped. A negative control slide (incubation without enzyme solution) and positive control slide (induction of DNA strand breaks using recombinant DNase I) as recommended by the manufacturer were included in all experimental runs. TUNEL-positive cells were quantified in one hemisphere by a blinded observer in two section planes including hippocampus, thalamus, cortical gray and underlying white matter (bregma −1,76 mm), and caudate-putamen (bregma 1,05 mm). The Allen Mouse Brain Coronal Atlas (available from http://brain-map.org) was used as an anatomical reference for brain structures. Cell counts represent absolute numbers of cells in the respective brain areas. Data are presented as median number of labeled cells per area and interquartile range (IQR).

Excitotoxic brain injury model and treatment

Brain lesions were induced using an ibotenate-based animal model of newborn excitotoxic brain injury as described in detail previously (Marret et al., 1995; Neubauer et al., 2016). In brief, five-day-old (P5) CD-1 mouse pups were anesthetized using isoflurane (2−3 vol%, with 2 L/min oxygen as a carrier gas) and prone-positioned under a stereotactic device (Stoelting Europe, Dublin, Ireland). Intracranial (i.c.) injections were performed with a 25 G needle attached to a 50 µl Hamilton syringe mounted on a calibrated microdispenser. The needle was inserted stereotactically in the fronto-parietal area of the right hemisphere – 2 mm dextrolateral to the midline in the lateral-medial plane and 3 mm rostral to the intersection of the sagittal and lambdoid sutures in the rostro-caudal plane. The tip of the needle was inserted 2 mm deep and a single bolus (1 µl) of ibotenate (5 µg/µl) was injected. To ensure distribution and avoid leakage, the needle tip was left in place for 30 s and then partially withdrawn. A second bolus was subsequently applied more superficially (1 mm insertional depth). After another 30 s, the needle was removed completely. Pups could recover from anesthesia under a warming lamp and were then returned to their dam until further interventions or sacrification.

One hour after the excitotoxic insult, animals were randomly assigned to the aforementioned treatment groups and received a single i.p. injection of LMP or equal amounts of vehicle NaCl 0.9 %.

Lesion size determination

Determination of lesion size in cortical gray and underlying white matter as a primary study endpoint was carried out as described previously (Griesmaier et al., 2012). Animals were sacrificed 24 h (P6) or 120 h (P10) after i.c. ibotenate injection. Brains were formaldehyde-fixed, paraffin-embedded, sliced serially into 10 µm-thick coronal sections and stained with cresyl violet acetate. The maximum rostro-caudal lesion diameter (number of sections with present lesion multiplied by section thickness), which correlates with the stereologically determined lesion volume (Rangon et al., 2007), served as an indicator of lesion extension. Lesion sizes were determined by two independent blinded observers. Data are shown as median length of the lesion in the rostro-caudal axis [µm] and IQR.

Immunohistochemistry for cleaved caspase-3 and isolectin B4

For evaluation of immunohistochemical endpoints, slides were processed as described previously (Neubauer et al., 2016). Sections adjacent to the lesion were deparaffinized in xylene and passed through graded alcohol series. To quench intrinsic peroxidase activity, immunohistochemical slides were immersed in 2 % H_2O_2/methanol for 30 min. Antigen retrieval was achieved by heating slides in a 10 mM citrate buffer (pH 6.0) for 15 min in a microwave oven. After non-specific blocking with biotin-free 1 % bovine serum albumin in tris-buffered saline containing 0.5 % Triton X-100/0,1 % cold water fish skin gelatine/0.05 % Tween-20, sections were incubated overnight at 4 °C with the following antibody dilutions: rabbit polyclonal anti-cleaved caspase-3 antibody (1:200) or biotinylated isolectin B4 (1:60). Sections were rinsed with PBS, and afterwards incubated for 90 min at 25 °C with biotinylated goat anti-rabbit IgG (1:200). After rinsing with PBS, sections were incubated for 45 min at 25 °C with avidin-biotin complex (Vectastain Elite ABC Kit). Visualization was performed using diaminobenzidine as enzyme substrate. After removing excess enzyme substrate, sections were dehydrated, cleared in xylene and cover-slipped. In the case of isolectin B4, sections were directly incubated with avidin-biotin complex reagents and detected using diaminobenzidine. Specificity of staining was ensured by routine inclusion of a negative control in all experimental runs.

Immunohistochemical quantification

Immunohistochemical quantification was conducted by a blinded observer as follows: Cleaved caspase-3-positive and isolectin B4-positive activated microglia were quantified in a single section plane in cortical gray and adjacent white matter at the level of the ibotenate-induced lesion in the damaged right (ipsilateral) hemisphere and in the corresponding area of the undamaged (contralateral) left hemisphere. Perilesional areas were defined as 1000 µm x pallial thickness. Cell counts represent absolute numbers of cells in the respective brain areas. Data are presented as median number of labeled cells per area and IQR.

Data and statistical analyses

Statistical analyses were performed using SPSS Statistics Software, version 24.0, for Windows (SPSS Inc. Chicago, IL, USA). Data distribution was evaluated by means of histogram analysis and the Shapiro-Wilk Test. For data not belonging to a particular distribution, a Mann-Whitney *U* test was applied for comparisons between two groups. If more than two groups were compared at a time, overall differences between groups were detected with a Kruskal-Wallis Test. If applicable, a post hoc analysis was conducted by means of a Mann-Whitney *U* test with Bonferroni correction for multiple comparisons. Results were regarded as statistically significant when $p < 0.05$.

Results

In vitro experiments

LMP reduces cell viability in uninjured, but not glutamate-exposed cells

First, we analysed the effect of LMP on cell viability in mouse hippocampal neuronal HT-22 cells that were either exposure-naïve or exposed to glutamate. Kruskal-Wallis Test revealed a significant overall difference in cell viability ($H_{(11)} = 25.019$, $p = 0.009$; $n = 48$). In cells not exposed to glutamate, pairwise comparisons showed a significant decrease in cell viability following treatment with 50 µM LMP in comparison to untreated controls ($U = 27.500$, $p = 0.030$; Mann-Whitney *U* test, significance Bonferroni-corrected). Exposure to glutamate led to a significant reduction in cell viability in comparison to unexposed controls ($U = 30.250$, $p = 0.012$; Mann-Whitney *U* test, significance Bonferroni-corrected), but no significant differences in cell viability between LMP- and untreated cells were detected (all $p > 0.05$; Mann-Whitney *U* test, significances Bonferroni-corrected) (Fig. 1). Details are provided in Table 1. Representative images of HT 22 cells are shown in Fig. 2.

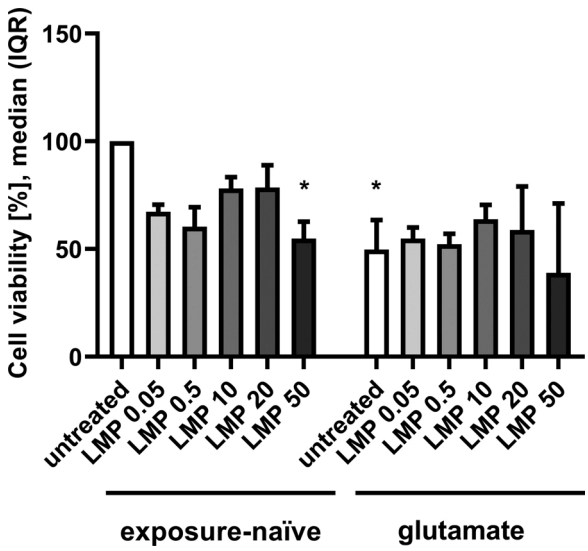

Fig. 1. Effect of levomepromazine (LMP) on cell viability in exposure-naïve and glutamate-exposed HT-22 cells. Cells were either untreated or pre-treated with levomepromazine (LMP) prior to glutamate exposure. Data are displayed as median and interquartile range (IQR), *p < 0.05.

Table 1
Cell viability in uninjured (exposure-naïve) and injured (glutamate-exposed) HT-22 cells following levomepromazine (LMP) treatment. HT-22 cells were treated with five different concentrations of LMP or were left untreated and were subsequently either returned to standard conditions or exposed to glutamate. Cell viability was assessed colorimetrically. Untreated, unexposed cells served as a viability control. All samples were analyzed in quintuplicates. Findings were confirmed in four independent subsets of experiments. IQR, interquartile range; LMP, levomepromazine; n.a., not applicable.

Group	Cell viability [%], median (IQR)	
	Exposure-naïve	Glutamate-exposed
Untreated	100.0 (n.a.)	49.7 (45.3; 63.5)
LMP 0.05 μM	67.2 (58.8; 70.6)	54.8 (36.8; 60.0)
LMP 0.5 μM	60.4 (47.9; 69.5)	52.3 (36.1; 57.1)
LMP 10 μM	78.1 (61.9; 83.4)	63.8 (44.6; 70.5)
LMP 20 μM	78.5 (68.0; 88.9)	58.9 (41.2; 79.1)
LMP 50 μM	54.8 (48.0; 62.7)	38.9 (27.8; 71.2)

In vivo experiments

Study population

A total number of 219 animals were used for the study. 7 animals were excluded because they did not meet the body weight inclusion criterion, 212 were included in the study and randomized to treatment groups (toxicity study in healthy animals: n = 39; excitotoxic injury plus treatment: n = 173). In the toxicity study, none of the animals died prior to endpoint analyses. In the excitotoxic injury plus treatment group, 8 animals died immediately after i.c. insult without receiving any i.p. injection. 4 animals died after i.p. injection (2 mice receiving LMP 0.5 μg/g bw and 1 mouse receiving 1 μg/g bw died one day after intervention; 1 mouse receiving LMP 0.05 μg/g bw died two days after intervention). The remainder were randomized to endpoint analyses on P6 (n = 79; male: n = 41/female: n = 38) or P10 (n = 82; male: n = 44/female: n = 38). None of the animals reached humane endpoint criteria with the need to be euthanized.

Toxicity study in healthy animals

Administration of LMP reduces body weight gain, but not brain weight in healthy animals. Kruskal-Wallis Test showed a significant overall difference in relative body weight gain from P5 to P6 in healthy animals treated with vehicle or LMP ($H_{(4)} = 22.099$, $p < 0.001$; n = 39). Post hoc pairwise multiple comparisons showed significantly less relative body weight gain following application of LMP 1 μg/g bw in comparison to vehicle (U = 20.625, p = 0.003) and to lower-dose LMP treatments (LMP 1 μg/g bw vs. LMP 0.05 μg/g bw: U = 21.375, p = 0.002; LMP 1 μg/g bw vs. LMP 0.1 μg/g bw: U = 19.500, p = 0.006; Mann-Whitney *U* test, significances Bonferroni-corrected). Details on weight development are shown in Fig. 3.

No statistically significant overall differences in brain weight were detected on P6 ($H_{(4)} = 3.704$, p = 0.447, n = 39; median (IQR), [g]; vehicle: 0.18 (0.17; 0.19); LMP 0.05 μg/g bw: 0.18 (0.15; 0.19); LMP 0.1 μg/g bw: 0.18 (0.15; 0.19); LMP 0.5 μg/g bw: 0.18 (0.16; 0.20); LMP 1.0 μg/g bw: 0.19 (0.18; 0.20)).

LMP treatment does not induce cell death in healthy animals. In order to determine potential negative effects of LMP on the healthy developing brain, in situ detection of DNA fragmentation as occurring in cell death was performed by means of a TUNEL assay. 24 h after i.p. injection of LMP or vehicle, no statistically significant overall differences in the number of TUNEL-positive cells were observed in gray matter, white matter, hippocampus, thalamus or caudate-putamen (Kruskal-Wallis Test: all $p > 0.05$, n = 33–34). Details can be found in Table 2. Representative photomicrographs of TUNEL staining are shown in Fig. 4.

Effects of LMP following excitotoxic injury

Administration of LMP reduces body weight gain, but not brain weight in animals suffering from excitotoxic brain injury. In animals subjected to excitotoxic injury, Kruskal-Wallis Test showed a significant overall difference in relative body weight gain from P5 to P6 ($H_{(4)} = 55.335$, $p < 0.001$; n = 161) and P5 to P10 ($H_{(4)} = 10.946$, p = 0.027; n = 82). Post hoc pairwise multiple comparisons showed significantly less relative body weight gain from P5 to P6 following application of LMP 1 μg/g bw in comparison to vehicle (U = 74.188, $p < 0.001$) and to lower-dose LMP treatments (LMP 1 μg/g bw vs. LMP 0.05 μg/g bw: U = 63.844, $p < 0.001$; LMP 1 μg/g bw vs. LMP 0.1 μg/g bw: U = 65.730, $p < 0.001$; LMP 1 μg/g bw vs. LMP 0.5 μg/g bw: U = 34.229, p = 0.036; Mann-Whitney *U* test, significances Bonferroni-corrected) as well as LMP 0.5 μg/g bw in comparison to vehicle (U = 39.959, p = 0.007, Mann-Whitney *U* test, significances Bonferroni-corrected). Concerning relative body weight gain from P5 to P10, significance was lost with pairwise comparisons. Details on weight development are shown in Fig. 5.

With regard to brain weight, Kruskal-Wallis Test showed a significant overall difference on P6 ($H_{(4)} = 11.707$, p = 0.020, n = 79; median (IQR), [g]; vehicle: 0.19 (0.18; 0.20); LMP 0.05 μg/g bw: 0.19 (0.17; 0.20); LMP 0.1 μg/g bw: 0.18 (0.18; 0.19); LMP 0.5 μg/g bw: 0.17 (0.16; 0.18); LMP 1.0 μg/g bw: 0.18 (0.16; 0.18)), but significance was lost with pairwise comparisons (all $p > 0.05$; Mann-Whitney *U* test, significances Bonferroni-corrected). On P10, no significant overall differences in brain weight were detected ($H_{(4)} = 1.926$, p = 0.749; n = 82; Kruskal-Wallis Test; median (IQR), [g]; vehicle: 0.25 (0.24; 0.27); LMP 0.05 μg/g bw: 0.25 (0.24; 0.27); LMP 0.1 μg/g bw: 0.25 (0.24; 0.27); LMP 0.5 μg/g bw: 0.25 (0.24; 0.26); LMP 1.0 μg/g bw: 0.25 (0.23; 0.26)).

LMP treatment does not affect excitotoxic lesion size

Following excitotoxic injury, no significant overall differences in lesion sizes were detected when evaluated 24 h (P6; gray matter: $H_{(4)} = 1.941$, p = 0.747, n = 71; white matter: $H_{(4)} = 4.276$, p = 0.370, n = 71; Kruskal-Wallis Test; 8 samples excluded due to methodological reasons) or 120 h after excitotoxic insult (P10; gray matter: $H_{(4)} = 4.137$, p = 0.388, n = 72; white matter: $H_{(4)} = 2.230$, p = 0.694, n = 72; Kruskal-Wallis Test; 10 samples excluded due to methodological reasons). Details can be found in Table 3. Representative photomicrographs of cresyl violet-stained sections showing excitotoxic lesions can

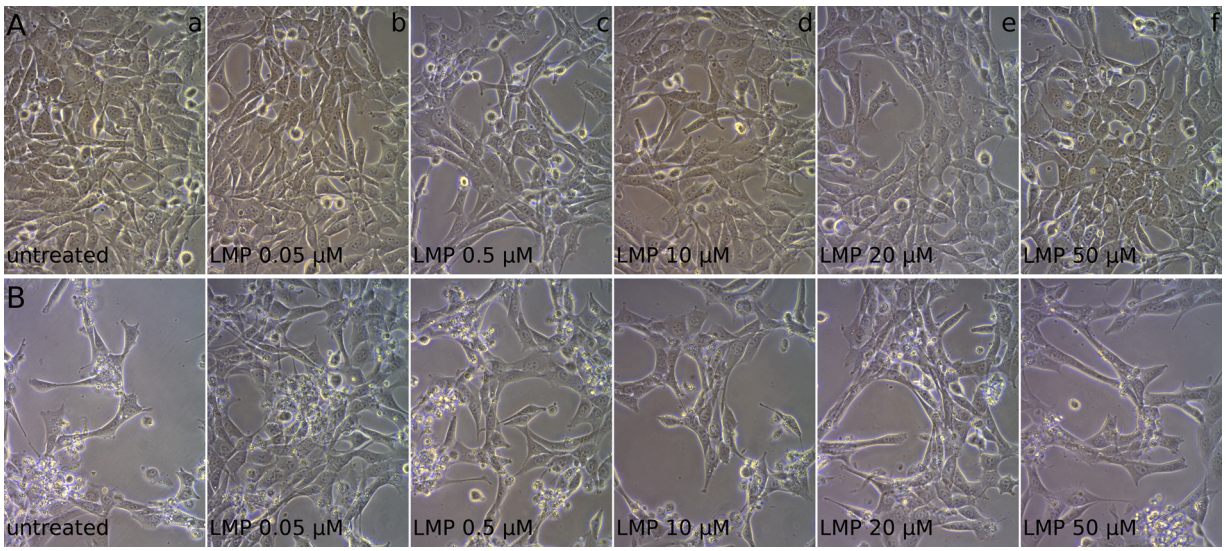

Fig. 2. Representative images of exposure-naïve HT-22 cells (A) and HT-22 cells exposed to glutamate (B). Cells were either untreated (columns a) or pre-treated with levomepromazine (LMP) 0.05 µM (columns b), 0.5 µM (columns c), 10 µM (columns d), 20 µM (columns e), 50 µM (columns f) prior to glutamate exposure. Phase contrast microscopy, 200-fold magnification.

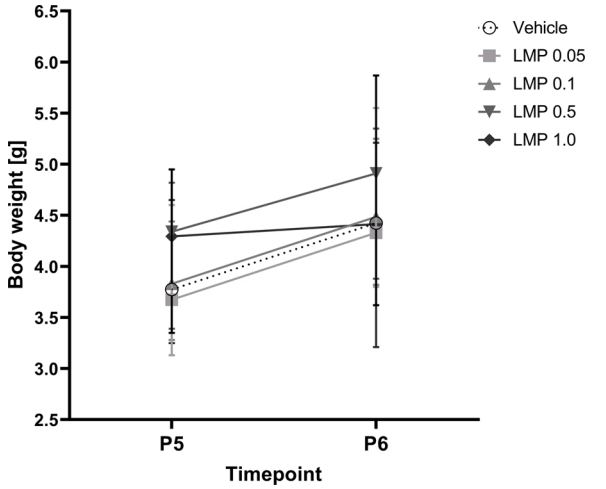

Fig. 3. Effect of levomepromazine (LMP) on body weight (bw) development in healthy animals. Healthy, uninjured animals received a single intraperitoneal injection of LMP or vehicle NaCl 0.9 % on postnatal day 5 (P5). Body weight in grams (g) was determined with medical precision scales on P5 and P6. Data are displayed as median as well as upper and lower limits (minimum/maximum). Numbers represent dosages in µg/g bw. Number of animals per group: n = 7-8. LMP, levomepromazine.

be found in Fig. 6.

LMP treatment does not affect excitotoxicity-induced apoptotic cell death

Evaluation 24 h after insult indicated no significant overall differences in the number of activated caspase-3-positive cells in perilesional gray or adjacent white matter (ipsilateral gray matter: $H_{(4)} = 2.639$, $p = 0.620$, $n = 25$; ipsilateral white matter: $H_{(4)} = 1.395$, $p = 0.845$, $n = 25$; Kruskal-Wallis Test).

In corresponding areas of the undamaged left hemisphere, no statistically significant overall differences were found in the number of activated caspase-3-positive cells (contralateral gray matter: $H_{(4)} = 5.084$, $p = 0.279$, $n = 25$; contralateral white matter: $H_{(4)} = 3.892$, $p = 0.421$, $n = 25$; Kruskal-Wallis Test).

Details are presented in Table 4. Representative photomicrographs of activated caspase-3 immunohistochemistry can be found in Fig. 7.

LMP treatment does not affect microglial cell activation

As microglial cells are known to play a crucial role in excitotoxic neonatal brain injury, microglial cell activation was assessed by means of isolectin B4 staining (Dommergues et al., 2003).

No significant overall differences in the number of activated isolectin B4-positive cells were detected in perilesional gray or adjacent white matter when evaluated 24 h (gray matter: $H_{(4)} = 1.743$, $p = 0.783$, $n = 25$; white matter: $H_{(4)} = 2.932$, $p = 0.569$, $n = 25$; Kruskal-Wallis Test) or 120 h after excitotoxic insult (gray matter: $H_{(4)} = 3.618$, $p = 0.460$, $n = 25$; white matter: $H_{(4)} = 4.106$, $p = 0.392$, $n = 25$;

Table 2
Effect of levomepromazine (LMP) treatment on cell death in the healthy developing brain. 5-day-old (P5) healthy, uninjured mouse pups received a single intraperitoneal injection of LMP or vehicle NaCl 0.9 %. Cell death as indicated by DNA fragmentation was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) on postnatal day 6 (P6). Cell counts represent absolute numbers of cells in the respective brain areas. Number of animals per group: n = 6–7. IQR, interquartile range; LMP, levomepromazine.

Group	TUNEL-positive cells, median (IQR)				
	Gray matter	White matter	Hippocampus	Thalamus	Caudate-Putamen
Vehicle	5.7 (5.3; 8.3)	7.3 (5.8; 8.3)	8.3 (5.0; 10.5)	6.0 (5.0; 6.3)	7.3 (5.7; 16.5)
LMP 0.05 µg/g bw	9.0 (4.7; 10.3)	8.5 (6.5; 10.5)	9.0 (6.0; 11.5)	7.0 (4.0; 9.8)	7.9 (6.3; 10.3)
LMP 0.1 µg/g bw	6.0 (2.3; 10.3)	4.7 (4.0; 10.0)	7.8 (6.7; 12.3)	6.3 (5.8; 8.3)	8.0 (5.5; 15.0)
LMP 0.5 µg/g bw	8.1 (6.1; 10.6)	8.6 (6.3; 12.0)	10.0 (8.3; 12.6)	7.4 (6.1; 9.4)	9.3 (5.0; 14.0)
LMP 1 µg/g bw	6.5 (6.0; 7.0)	8.3 (5.0; 10.7)	11.3 (9.0; 13.0)	7.8 (3.3; 9.0)	10.3 (6.4; 11.1)
Kruskal-Wallis Test	$H_{(4)} = 3.158$ $p = 0.532$	$H_{(4)} = 2.559$ $p = 0.634$	$H_{(4)} = 3.957$ $p = 0.412$	$H_{(4)} = 2.014$ $p = 0.733$	$H_{(4)} = 0.513$ $p = 0.972$

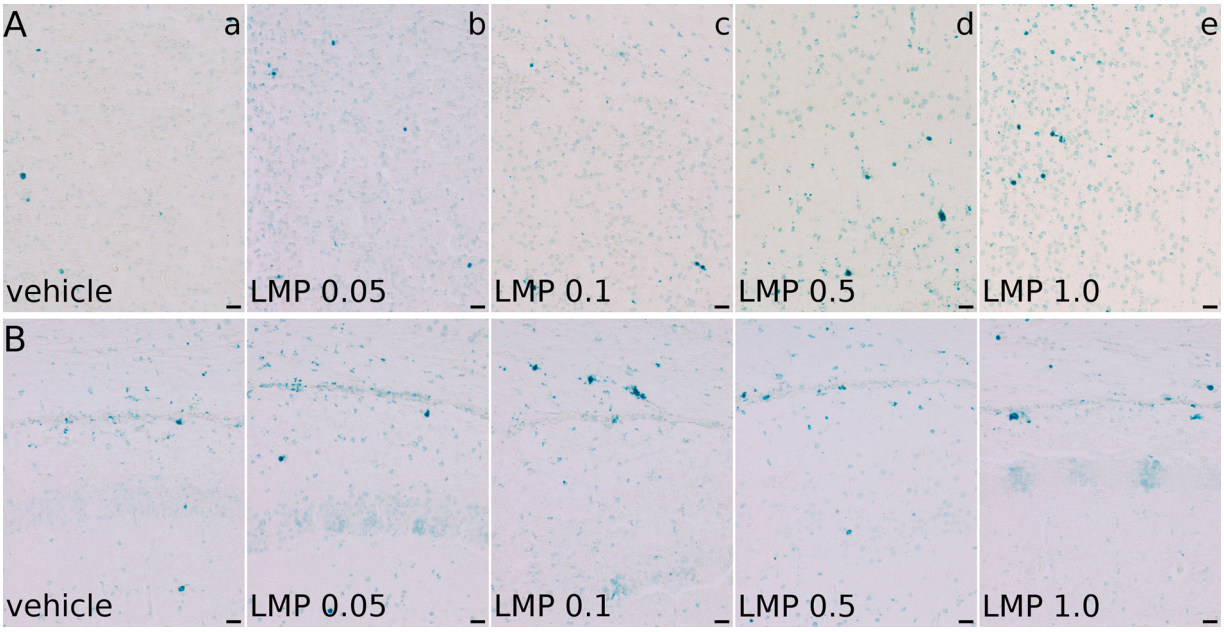

Fig. 4. Representative images of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining in caudate-putamen (A) and hippocampus (B). 5-day-old (P5) healthy mouse pups received a single i.p. injection of vehicle NaCl 0.9 % (columns a), or LMP in a dosage of 0.05 μg/g body weight (bw) (columns b), 0.1 μg/g bw (columns c), 0.5 μg/g bw (columns d), or 1 μg/g bw (columns e). DNA fragmentation was detected by TUNEL assay on P6. Visualization was performed using a 200-fold magnification (scale bar = 20 μm).

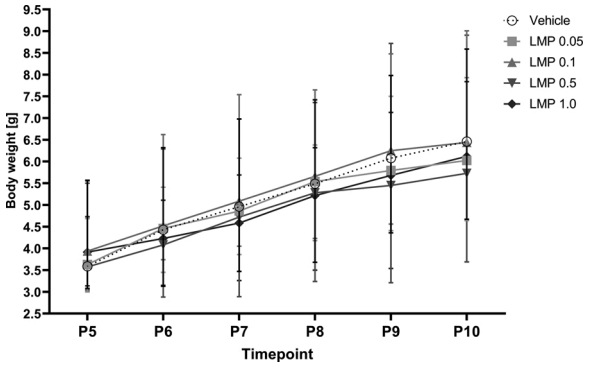

Fig. 5. Effect of levomepromazine (LMP) on body weight (bw) development in animals suffering from excitotoxic brain injury. Excitotoxic brain injury was induced on postnatal day 5 (P5). One hour after insult, animals received a single intraperitoneal injection of LMP or vehicle NaCl 0.9 %. Body weight (bw) in grams (g) was determined with medical precision scales on a daily basis from P5 to P10. Data are displayed as median as well as upper and lower limits (minimum/maximum). Numbers represent LMP dosages in μg/g bw. Number of animals followed from P5 to P6: n = 15–17 per group. Number of animals followed from P5 to P10: n = 16–17 per group. LMP, levomepromazine.

Table 3
Effect of levomepromazine (LMP) treatment on excitotoxic brain injury extent. Excitotoxic brain injury was induced on postnatal day 5 (P5). One hour after insult, animals received a single intraperitoneal injection of LMP or vehicle NaCl 0.9 %. Lesion size was determined 24 h (P6) or 120 h (P10) after insult on cresyl violet-stained coronal brain sections in gray and adjacent white matter. Number of animals per group: n = 12-17. IQR, interquartile range; LMP, levomepromazine.

Group	Lesion size [μm], median (IQR			
	P6		P10	
	Gray matter	White matter	Gray matter	White matter
Vehicle	720 (620; 900)	360 (320; 520)	960 (740; 1240)	620 (390; 1040)
LMP 0.05 μg/g bw	660 (550; 770)	360 (270; 500)	840 (760; 1040)	640 (480; 800)
LMP 0.1 μg/g bw	680 (540; 880)	400 (260; 500)	760 (700; 920)	480 (380; 900)
LMP 0.5 μg/g bw	720 (600; 760)	320 (240; 440)	800 (720; 1040)	700 (570; 800)
LMP 1 μg/g bw	760 (650; 790)	480 (380; 510)	860 (750; 960)	500 (460; 680)
Kruskal-Wallis Test	$H_{(4)} = 1.941$ $p = 0.747$	$H_{(4)} = 4.276$ $p = 0.370$	$H_{(4)} = 4.137$ $p = 0.388$	$H_{(4)} = 2.230$ $p = 0.694$

Kruskal-Wallis Test).

In corresponding areas of the undamaged left hemisphere no significant overall differences in the number of isolectin B4-positive activated microglial cells were detected on P6 (contralateral gray matter: $H_{(4)} = 1.538$, $p = 0.820$, $n = 25$; contralateral white matter: $H_{(4)} = 1.507$, $p = 0.825$, $n = 25$; Kruskal-Wallis Test). On P10, significant overall differences were detected in both gray and white matter (contralateral gray matter: $H_{(4)} = 12.606$, $p = 0.013$, $n = 25$; contralateral white matter: $H_{(4)} = 9.656$, $p = 0.047$, $n = 25$; Kruskal-Wallis Test). Post hoc pairwise comparisons showed significantly higher numbers of activated isolectin B4-positive microglial cells following treatment with LMP 0.1 μg/g bw in contralateral gray matter (LMP 0.1 μg/g bw vs. control; U=-13.700, $p = 0.012$; Mann-Whitney *U* test, significance Bonferroni-corrected). Significance was lost with pairwise comparisons for contralateral white matter (all $p > 0.05$; Mann-Whitney *U* test, significances Bonferroni-corrected). Details are presented in Fig. 8. Representative photomicrographs of isolectin B4 immunohistochemistry can be found in Fig. 9.

Discussion

For decades, management of stress and pain was disregarded in NICUs. Effective pain management has now gained importance, as an increasing body of evidence suggests that stress and painful stimuli early in neonatal life lead to impairments in brain growth, metabolism and functionality (Bhutta and Anand, 2002; Anand and Hickey, 1987; Hall and Anand, 2014). A pivotal mechanism in this context seems to be

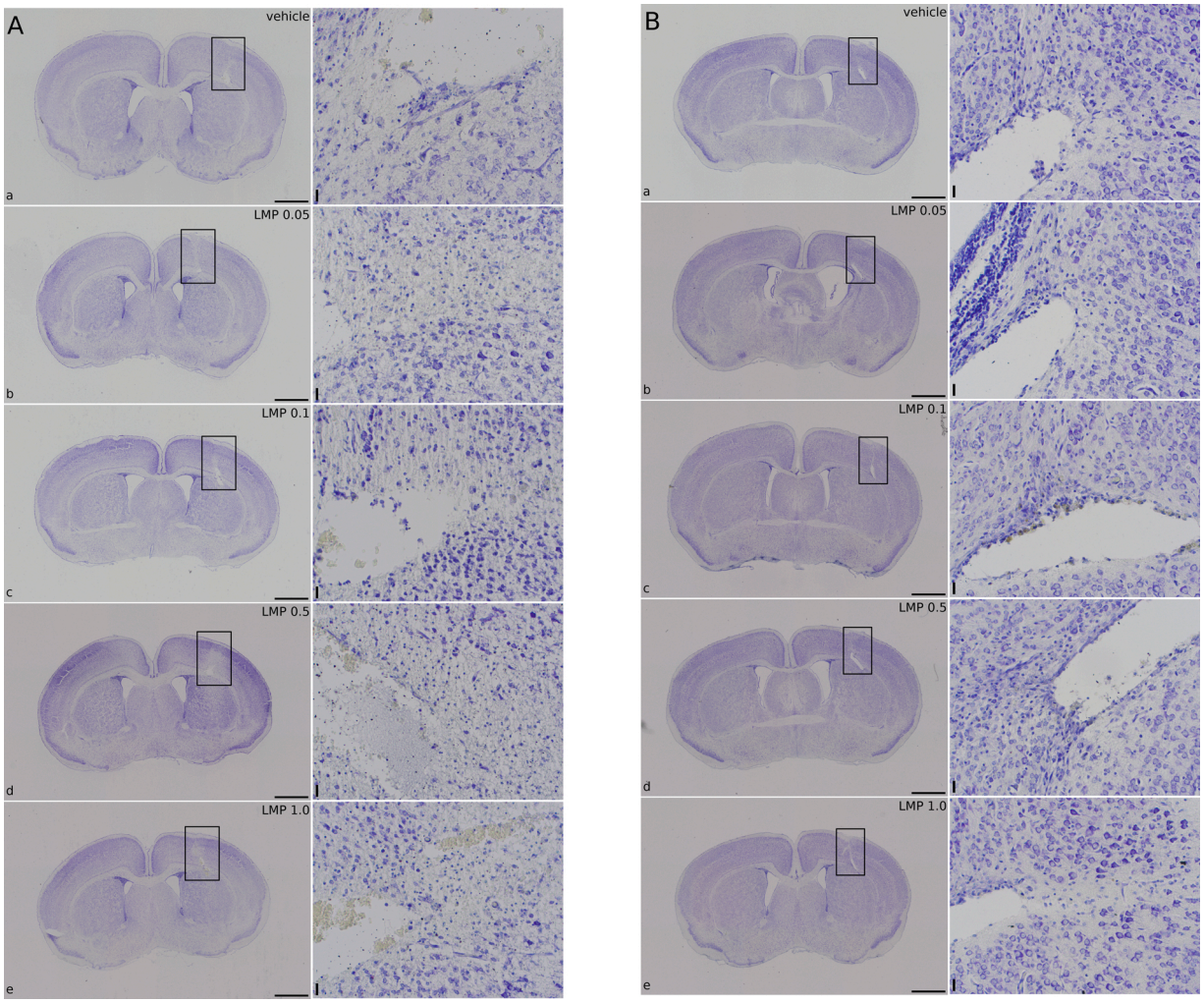

Fig. 6. Representative photomicrographs of cresyl violet-stained coronal brain sections showing excitotoxic lesions on postnatal day 6 (P6; Panel A, a-e) and P10 (Panel B, a-e). Excitotoxic brain injury was induced on postnatal day 5 (P5). One hour after insult, animals received a single intraperitoneal injection of vehicle NaCl 0.9 % (rows a), LMP 0.05 μg/g body weight (bw) (rows b), LMP 0.1 μg/g bw (rows c), LMP 0.5 μg/g bw (rows d), or LMP 1 μg/g bw (rows e). Whole-brain visualization (left side) was performed using a 40-fold magnification (scale bar = 1000 μm). Magnification boxes (right side) show a 200-fold magnification of the excitotoxic lesion in each treatment group, rotated 90° clockwise (scale bar = 20 μm).

Table 4
Effect of levomepromazine (LMP) treatment on excitotoxicity-induced apoptotic cell death. Excitotoxic brain injury was induced on postnatal day 5 (P5). One hour after insult, animals received a single intraperitoneal injection of LMP or vehicle NaCl 0.9 %. Immunohistochemical analyses of activated caspase-3 were performed 24 h after insult on P6 in gray and adjacent white matter of the injured right (ipsilateral) and contralateral left hemisphere. Number of animals per group: n = 4–6. IQR, interquartile range; LMP, levomepromazine.

Group	Activated caspase-3-positive cells, median (IQR)			
	Ipsilateral gray matter	Ipsilateral white matter	Contralateral gray matter	Contralateral white matter
Vehicle	18.7 (5.1; 36.1)	3.5 (1.1; 4.8)	2.3 (1.8; 2.7)	0.9 (0.4; 1.8)
LMP 0.05 μg/g bw	11.3 (4.9; 28.9)	2.0 (1.5; 5.1)	1.3 (1.0; 1.6)	1.0 (0.4; 1.1)
LMP 0.1 μg/g bw	19.3 (12.9; 30.0)	4.3 (2.8; 6.3)	2.8 (0.4; 3.9)	0.8 (0.2; 1.3)
LMP 0.5 μg/g bw	21.9 (15.3; 37.4)	2.3 (1.2; 8.5)	5.6 (1.3; 9.6)	2.0 (0.9; 3.1)
LMP 1 μg/g bw	27.3 (18.0; 31.3)	3.3 (2.6; 4.6)	1.8 (1.4; 3.0)	1.0 (0.0; 1.9)
Kruskal-Wallis Test	$H_{(4)} = 2.639$ $p = 0.620$	$H_{(4)} = 1.395$ $p = 0.845$	$H_{(4)} = 5.084$ $p = 0.279$	$H_{(4)} = 3.892$ $p = 0.421$

excitotoxicity, characterized by excessive release and impaired re-uptake of glutamate, ultimately leading to neurodegeneration and cell death (Vannucci and Hagberg, 2004). A pharmacologic class with the potential to counter these noxious effects are phenothiazines which have been shown to be anti-excitotoxic, anti-apoptotic and anti-oxidative (Geng et al., 2017; An et al., 2017; Varga et al., 2017; Gonzalez-Munoz et al., 2010). The long-standing phenothiazine LMP is currently experiencing a revival due to its favourable risk profile, flexible routes of administration, convenient dosing interval, as well as its potent sedative, analgesic and anaesthetic mechanisms of action (van der Zwaan et al., 2012; Hohl et al., 2013). These properties render it particularly attractive for use in NICUs. The safety of its administration in preterm infants, however, has not yet been investigated.

To the best of our knowledge, this study is the first to evaluate the

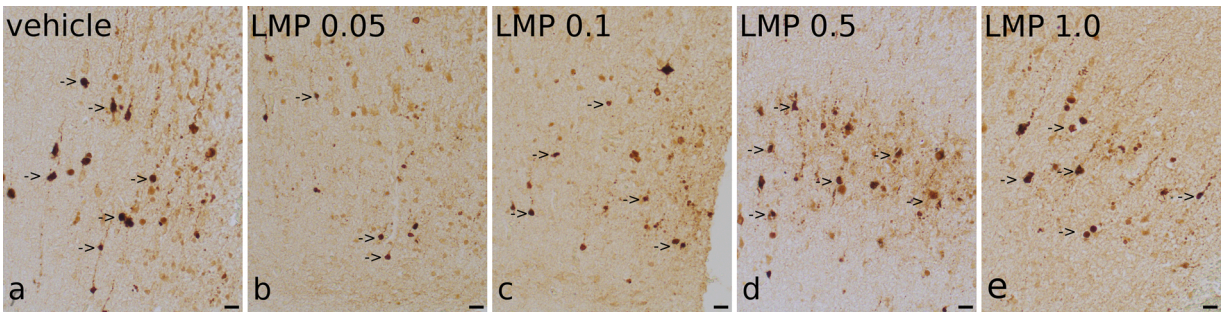

Fig. 7. Representative photomicrographs of activated caspase-3 immunohistochemistry. 5-day-old (P5) mouse pups were subjected to excitotoxic brain injury. One hour after insult, animals received a single i.p. injection of vehicle NaCl 0.9 % (a), or LMP in a dosage of 0.05 μg/g body weight (bw) (b), 0.1 μg/g bw (c), 0.5 μg/g bw (d), or 1 μg/g bw (e). Animals were sacrificed on P6. Photomicrographs show activated caspase-3-positive cells in ipsilateral perilesional gray matter. Visualization was performed using a 200-fold magnification (scale bar = 20 μm). Arrows indicate activated caspase-3 positive cells.

effects of LMP in both the healthy and the injured developing brain by use of established *in vitro* and *in vivo* models.

In vitro experiments

The effects of LMP were first assessed in an *in vitro* setting using the immortalized mouse hippocampal cell line HT-22, which is frequently used as a model system to study glutamate-induced toxicity in neuronal cells (Brimson et al., 2018; Sukprasansap et al., 2017; Breyer et al., 2007; Davis and Maher, 1994; Morimoto and Koshland, 1990).

In cells not exposed to glutamate, cell viability was significantly decreased following treatment with LMP in a dosage of 50 μM in comparison to untreated controls. No statistically significant differences in cell viability were observed with other LMP dosages in exposure-naïve HT-22 cells or in HT-22 cells exposed to glutamate. As the dosage of 50 μM can be considered high, non-specific effects are more likely to occur: Haenisch et al. reported an inhibition of organic cation transporters expressed in the brain at high-range LMP concentrations exceeding 20 μM (Haenisch et al., 2012). These effects may be masked in cases of glutamate toxicity, where ion fluxes are per se deranged. In a clinical setting, high-range concentrations may not be reached with recommended dosages. However, cautious use is still warranted, especially as LMP is known to accumulate in the brain with high brain-to-blood concentration ratios and long elimination half-life from brain tissue (Kornhuber et al., 2006a, b).

In vivo experiments

Toxicity study

To the best of our knowledge, no studies on the potential neurotoxic effects of LMP on the preterm brain have been conducted. Based on our preliminary *in vitro* analysis, the next step was to perform an *in vivo* toxicological study. The experiment used 5-day-old (P5) healthy, uninjured mouse pups, comparable to a gestational age of 24–32 weeks in human terms and coinciding with a particularly vulnerable phase of the developing brain (Clowry et al., 2014). LMP dosages were chosen according to pediatric standards (van der Zwaan et al., 2012; Hohl et al., 2013). As a somatometric endpoint, body weight development following LMP administration was monitored. A significantly lower relative body weight gain was observed in animals receiving the highest dosage (LMP 1 μg/g bw) in comparison to vehicle and lower-dose LMP treatments, while brain weight did not differ between groups. This may be due to a lower food intake in more deeply sedated animals (Owens, 2012). In our study, general wellbeing of experimental animals was not altered by pharmacologic treatment. Unfortunately, food intake was not measured separately, but a particular focus on weight development and caloric intake seems prudent in case of a potential clinical use of LMP.

In order to rule out potential neurotoxic effects of LMP on the heathy developing brain, in situ cell death detection was performed by a TUNEL assay. As LMP is known to accumulate in the brain in a region-specific distribution (Kornhuber et al., 2006a), various brain areas were evaluated. No statistically significant overall differences in the number of TUNEL-positive cells were observed between groups in gray matter, white matter, hippocampus, thalamus or caudate-putamen, indicating that LMP does not induce DNA fragmentation in these areas.

Effects of LMP following excitotoxic injury

Bearing in mind the neuroprotective effects of structurally related drugs in adult brain injury models (Geng et al., 2017; Songarj et al., 2015), the next step was to assess LMP in an animal model of neonatal excitotoxic brain injury. Owing to its long biological half-life (Dahl, 1976), a single i.p. injection of LMP was applied one hour after i.c. insult in the same concentrations used in the preceding toxicological study. Both short- (P6) and longer-term (P10) endpoints were included.

With regard to weight development, a significant reduction in relative body weight gain from P5 to P6 was observed following application of both LMP 1 μg/g bw and LMP 0.5 μg/g bw in comparison to vehicle, indicating a potential aggravation of the effects observed in healthy animals by the excitotoxic insult.

With regard to lesion size, no significant differences were detected between groups neither 24 nor 120 h after insult. Also, LMP did not affect caspase-3 activation 24 h after insult neither in the damaged ipsilateral nor in the undamaged contralateral hemisphere. With regard to microglial cell activation, no significant differences were detected on P6 or P10 in the damaged ipsilateral hemisphere. In contrast to previous studies reporting neuroprotection through phenothiazine administration, LMP thus failed to produce a neuroprotective effect. Potential reasons for this could be molecular differences between the substances used: Several *in vitro* studies using phenothiazine derivatives linked a side chain located at the 10-nitrogen also present in LMP to a significant reduction in anti-oxidative potential (Yu et al., 1992; Poteet et al., 2012). A neuroprotective effect could therefore be achieved through higher LMP concentrations - an approach also chosen by Geng et al. using chlorpromazine and promethazine in a rat stroke model (Geng et al., 2017). This however, might be outweighed by adverse effects, as indicated by the reduced viability observed in HT-22 cells and the reduced weight gain in animals treated with higher LMP doses in our study. In addition, LMP's accumulation in brain tissue and long elimination half-life need to be considered in case of higher-dose treatment, with possibly prolonged side effects (Kornhuber et al., 2006b). Interestingly, microglial cell activation on P10 was significantly higher in contralateral gray matter in animals treated with medium-dose LMP. This is in accordance with a study in Wistar rats reporting an increase in microglial activation status and numbers in both gray and white matter following treatment with chlorpromazine (Dejanovic et al., 2016). However, absolute numbers in our study were small and the clinical relevance of this finding is questionable.

Of importance, we could not discern specific toxic effects of LMP

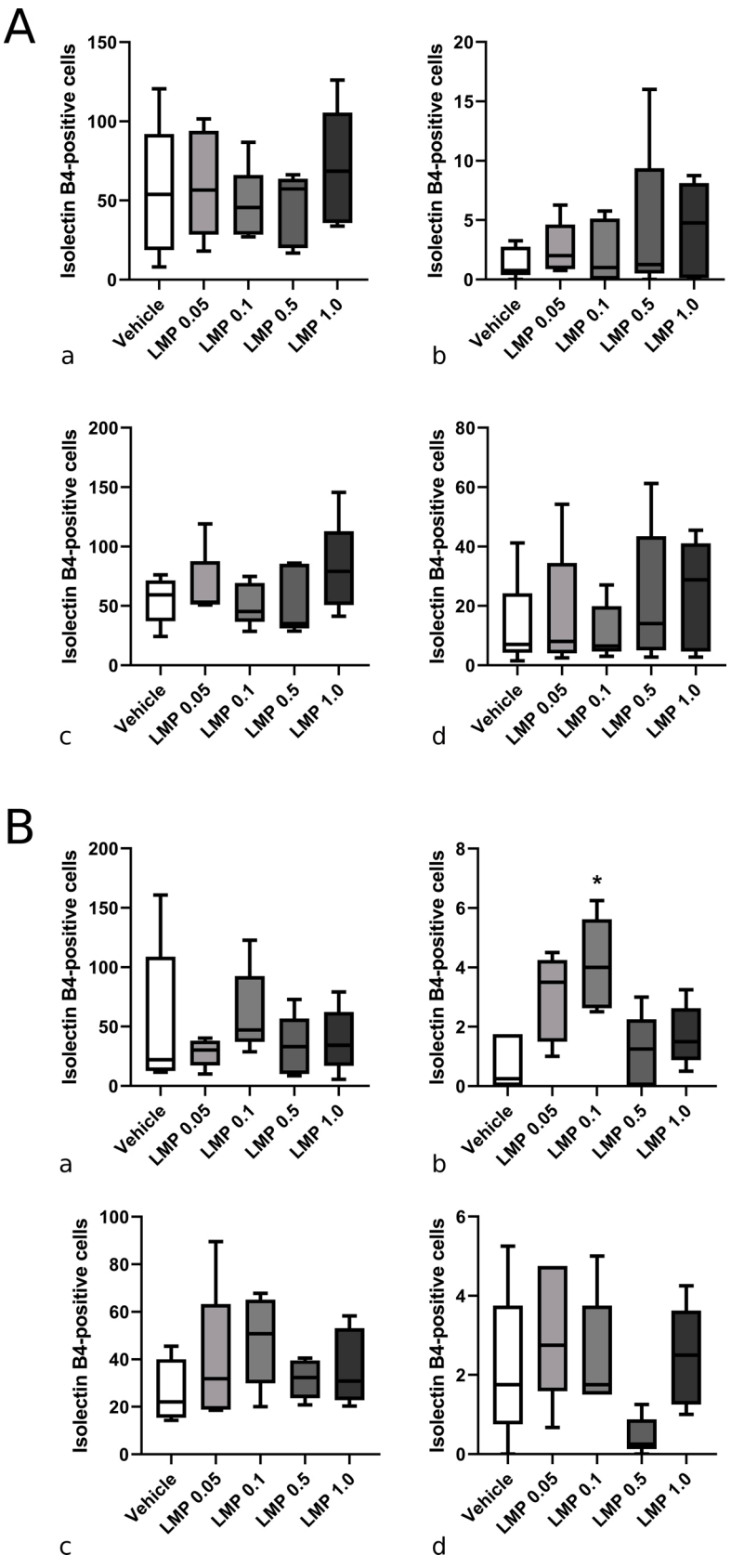

Fig. 8. Effects of levomepromazine (LMP) on microglial cell activation following excitotoxic brain injury. Excitotoxic brain injury was induced on postnatal day 5 (P5). One hour after insult, animals received a single intraperitoneal injection of vehicle NaCl 0.9 % or LMP. Isolectin B4 immunohistochemistry was conducted on postnatal day 6 (P6, Panel A) or 10 (P10, Panel B). Isolectin B4-positive activated microglial cells were quantified in ipsilateral gray (a) and white matter (c) as well as contralateral gray (b) and white matter (d). Numbers on y-axis represent cell counts in respective brain areas. Whiskers in box-and-whiskers plots represent 5th-95th percentiles. Numbers on x-axis represent LMP dosages in μg/g bw. Significantly higher numbers of activated isolectin B4-positive microglial cells were observed in contralateral gray matter following treatment with LMP 0.1 μg/g bw in comparison to vehicle-injected controls (post hoc Mann-Whitney *U* test with Bonferroni correction for multiple comparisons). *$p = 0.012$. Number of animals per group: $n = 5$.

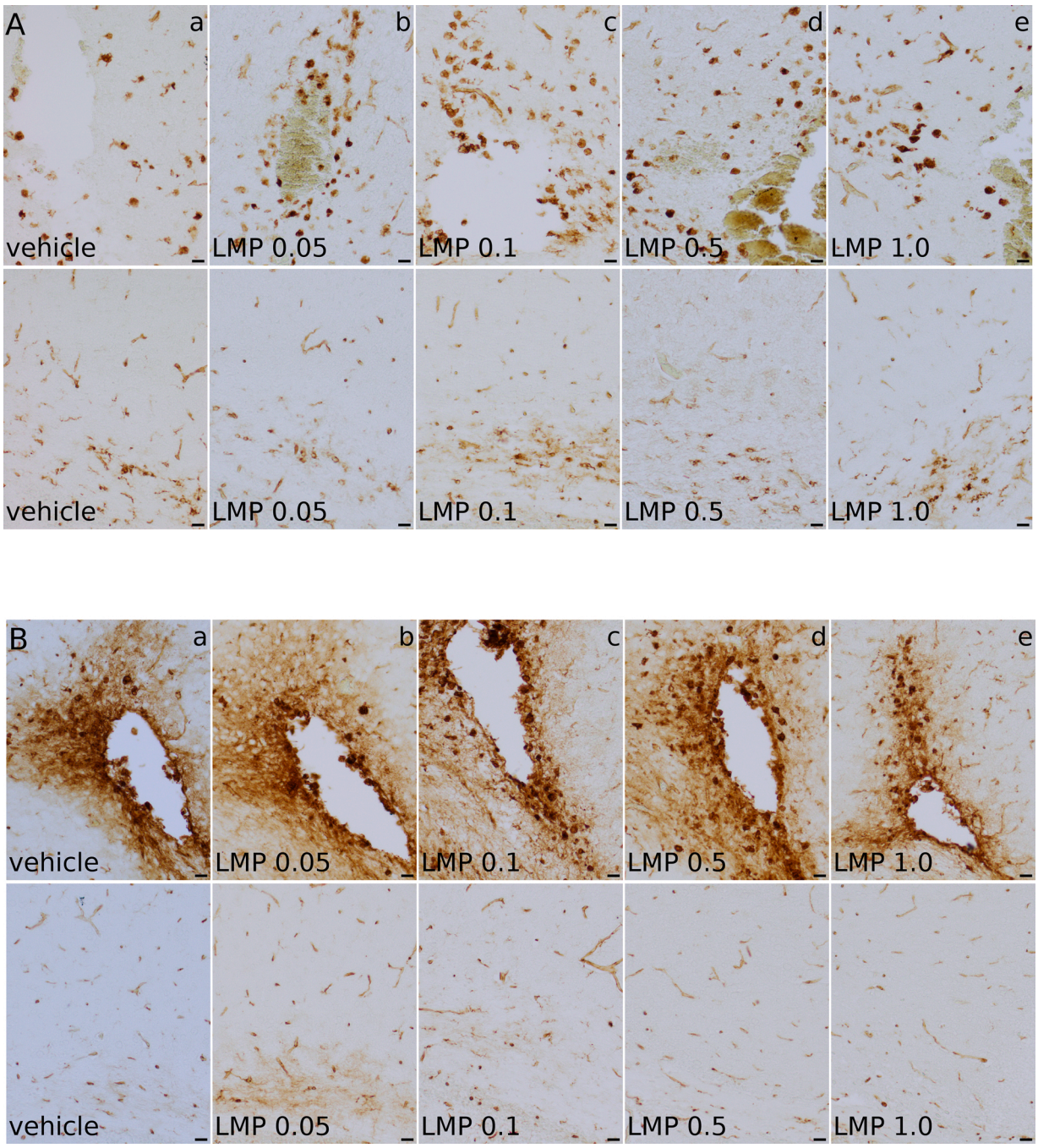

Fig. 9. Representative photomicrographs of isolectin B4 immunohistochemistry. 5-day-old (P5) mouse pups were subjected to excitotoxic brain injury. One hour after insult, they received a single i.p. injection of vehicle NaCl 0.9 % (columns a), or LMP in a dosage of 0.05 μg/g body weight (bw) (columns b), 0.1 μg/g bw (columns c), 0.5 μg/g bw (columns d), or 1 μg/g bw (columns e). Isolectin B4 immunohistochemistry was conducted on postnatal day 6 (P6, Panel A) or 10 (P10, Panel B). Photomicrographs show isolectin B4-positive activated microglial cells in ipsilateral right perilesional (upper rows) and contralateral left (lower rows) gray and white matter. Visualization was performed using a 200-fold magnification (scale bar = 20 μm).

treatment especially with the lower doses applied in our *in vivo* study. In particular, we did not observe enhanced glutamate-mediated neurotoxicity as described by Isom et al. in dopamine D_2 receptor antagonists such as LMP (Isom et al., 2013). A possible explanation is LMP's comparatively high affinity for and antagonistic activity at the serotonin 5-HT_{2A} receptor that might neutralize the effects of dopamine D_2 receptor antagonism through an inhibition of glutamate release (Isom et al., 2013; Lal et al., 1993). A more detailed elucidation of LMP's receptor binding activities in the developing brain was beyond the scope of this study, this needs to be addressed by future research.

To conclude, LMP failed to produce a protective effect in our *in vitro* and *in vivo* models of neonatal excitotoxic brain injury in contrast to previous studies reporting neuroprotection through phenothiazines. Specific toxic effects of LMP on the healthy and injured developing brain were not observed in our study, but its clinical use may be limited due to decreased weight gain especially in higher-dose treatments and its known pharmacokinetic properties.

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Ethics

Studies have been performed in compliance with ethical standards.

CRediT authorship contribution statement

Anna Posod: Conceptualization, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **Ira Winkler:** Writing - original draft, Writing - review & editing. **Karina Wegleiter:** Conceptualization, Investigation, Writing - original draft. **Eva Huber:** Methodology, Resources, Writing - review & editing. **Martina Urbanek:** Methodology, Resources, Writing - review & editing. **Ursula Kiechl-Kohlendorfer:** Supervision, Writing - review & editing. **Elke Griesmaier:** Conceptualization, Investigation, Supervision, Project administration, Visualization, Writing - original draft, Writing - review & editing.

Conflicts of interest

We declare no conflict of interest.

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